Research paper

Induction, Culture, and Taxane Production of Crown Galls and Hairy Roots of *Taxus sumatrana* (Miq) de Laub

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【Summary】

Taxus sumatrana (Miq) de Laub crown galls and hairy roots were induced by infecting stem and leaf segments with *Agrobacterium tumefaciens* strain A281, and *A. rhizogenes* strains AR1600 and ATCC15834. Results showed great variations in the transformation efficiencies among different *Agrobacterium* strains and *T. sumatrana* clones. Strain A281 had better transformation ability than strains AR1600 and ATCC15834 (42.3 vs. 15.1~19.2%). Among all 7 clones, clones E7 and NJR had the highest transformation efficiency of both crown gall and hairy root formation, whereas the lowest transformation rates occurred with clones E2 and J85 which developed no hairy roots when inoculated with strain AR1600. Some hairy roots formed callus tissues, like crown galls, which grew rapidly and produced taxanes when cultured on plant growth regulator-free medium. The integration of *Agrobacterium iaaM* and *rol B* genes into the crown gall and hairy root genome was verified with a PCR. After 40 d of culture, growth indexes (ratio of net fresh weight of cultures to initial weight) of crown galls, hairy roots, and hairy root calli (3.9~5.5) were higher than those of untransformed calli (2.5) and roots (0.31). Transformed crown galls and calli formed from hairy roots produced more 10-deacetyl baccatin III, baccatin III, and paclitaxel than did hairy roots. After 30 d of culture in liquid medium, growth indexes of crown galls and hairy roots were 8.13 and 6.03, respectively, which were better than those on solid medium, suggesting that this system has the potential for large-scale bioreactor culture in the future.

Key words: *Taxus sumatrana*, crown gall, hairy root, paclitaxel, taxanes.

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研究報告

南洋紅豆杉之腫瘤細胞及毛狀根誘導、培養與紫杉烷類生產

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摘 要

本研究係利用農桿菌A281與農桿叢根菌AR 1600與ATCC15834建立南洋紅豆杉(*Taxus sumatrana* (Miq) de Laub)莖段與葉片的腫瘤細胞與毛狀根培養。農桿菌的轉殖率在不同菌種與單株間有很大的差異，以A281誘導產生腫瘤的轉殖率(42.3%)高於AR1600與ATCC15834的毛狀根轉殖率(15.1~19.2%)。比較7個不同單株的轉殖率，不論在腫瘤或毛狀根，都以E7與NJR最高，E2與J85最低且於接種AR1600無法產生毛狀根。有些毛狀根會產生癒合組織，此癒合組織和腫瘤細胞一樣，可於不含植物生長調節劑的培養基快速生長與生產紫杉烷類化物。以PCR確認農桿菌的*iaaM*基因及農桿叢根菌的*rol B*基因已轉移至腫瘤與毛狀根。腫瘤細胞、毛狀根與毛狀根癒合組織在MS固體培養基培養40天，生長指數(培養結束之培植體淨生長鮮重對開始培養鮮重比)為3.9~5.5，高於未轉殖癒合組織(2.5)與根(0.31)。調查10-DB, baccatin III與paclitaxel的含量，以腫瘤細胞與毛狀根產生的癒合組織較高。將腫瘤細胞與毛狀根移入液體培養基中培養30天，其生長量為8.13與6.03，高於固體培養基培養，顯示具有利用生物反應器放大培養的潛力。

關鍵詞：臺灣紅豆杉、腫瘤細胞、毛狀根、紫杉醇、紫杉烷類。

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INTRODUCTION

Taxus sumatrana (Miq.) de Laub., a member of the Taxaceae family, has the widest distribution range among 11 species of the *Taxus* genus. It is found in the eastern Himalayas, southeastern China, Malaysia, and Taiwan (Li and Keng 1994). In Taiwan, it is distributed sparsely in mountains at around 2,000 m. Due to its superior timber quality, *T. sumatrana* belongs to 1st class conifers and is one of the 5 precious coniferous species in Taiwan.

Paclitaxel (or taxol), a complex diterpenoid, was originally isolated from *T. brevifolia*, and its structure was determined in 1971 (Wani et al. 1971). Since first approved by the US Food and Drug Administration for treating ovarian cancer in 1992 and metastatic breast cancer in 1994, paclitaxel has been widely used to treat patients with lung, neck, head, and leukemia cancers, and advanced forms of Kaposi's sarcoma (Rowinsky and Donehow 1995). Lately, it was reported that low-dose paclitaxel has a great potential for curing non-cancer diseases, such as skin disorders, renal and hepatic fibrosis, and inflammation, as well as benefitting axon regeneration, limb salvage, and coronary artery restenosis (Zhang et al. 2014). Consequently, demand for taxol is increasing worldwide. In addition to pacli-

taxel, more than 500 other diterpenoids were isolated from plants of the genus *Taxus* (Wang et al. 2011).

The original source of paclitaxel was the bark and branches of *Taxus* plants, which grow very slowly and contain only 0.01~0.045% (dry weight) of paclitaxel (Wang et al. 2011), thus limiting its supply. Much research focused on increasing paclitaxel production by other means (Tabata 2006). For example, the total synthesis of paclitaxel was successfully developed in 1994; however, the method is not practical because it took 28 steps while the yield rate was only 0.5% (Nicolaou et al. 1994). Paclitaxel and its related compounds can also be semi-synthesized from 10-deacetylbaccatin III (10-DB) or baccatin III (BC), natural products extracted from *Taxus* species (Ojima et al. 2014). Cell culture of different *Taxus* species is a promising alternative for paclitaxel production. Research on the selection of cell lines, calli, and cell cultures, improving techniques for taxane production, biotransformation strategies, and production via varied types of bioreactors has been widely explored (Malik et al. 2011, Khani et al. 2012, Nosov et al. 2014, Li et al. 2015). Crown galls (also called crown gall tumors or tumors) are induced by transformation with *Agrobacterium tumefacens*, while hairy roots are induced by *A. rhizogenes*. Both were found to be a potentially favorable method of producing high-value pharmaceuticals due to their stable and high productivity in plant growth regulator-free culture conditions with growth rates similar to those of the fastest growing cell suspension culture (Hu and Du 2006).

Han et al. (1994) first reported the successful genetic transformation of *Taxus* species using *A. tumefaciens* (C58 and Bo542 stains) to inoculate stems of *T. baccata* and *T. brevifolia* to form crown galls. Later, *Taxus* species hairy roots were successfully induced, including *T. x media* (Furmanowa and Skylowska-Baranek 2000, Skylowska-Baranek et al. 2009) and *T. cuspidata* (Kim et al. 2009). In other studies, *A. tumefaciens* was used to transfer the Gus gene into *T. x media* (Luan et al. 1996, Xu et al. 2012), *T. cuspidata*, and *T. chinensis* (Ketchum et al. 2007), and taxol synthesis-related genes into *T. sumatrana* (Ho et al. 2005) and *T. x media* (Exposito et al. 2010, Syklowska-Baranek et al. 2015). In addition to using *Agrobacterium*, a particle bombardment-mediated transient transformation system to transfer the Gus gene into *T. cuspidata* and *T. canadensis* was also developed (Vongpaseuth et al. 2007).

In this study, we aimed to establish an *Agrobacterium*-mediated transformation system for *T. sumatrana* and provide a stable supply of taxanes. The growth of transgenic cells and taxane concentrations were monitored to ensure that the system could be applied to large-scale bioreactor culture in the future.

MATERIALS AND METHODS

Plant material

Immature and mature fruits of *T. sumatrana*, collected from 7 mother trees in different mountain areas of Taiwan, were cleaned and sterilized after the pericarps were removed. Embryos, inside the seed coat and female gametophyte, were excised and placed on half-strength macroelements of MS medium (1/2MS; Murashige and Skoog 1962) containing 0.8 $g.L^{-1}$ polyvinylpyrrolidone (PVP) (Chang and Yang 1996). They were germinated and developed into whole plants about 3 cm tall in vitro after 10 wk in culture. Shoots about 0.5 cm long excised from these plants in vitro were subcultured monthly on 7.5 $g.L^{-1}$ agar solidified MS medium supplemented with 30 $g.L^{-1}$

sucrose and 1.5 g.L^{-1} activated charcoal (called M1 medium) and incubated at 25±2℃ under a 16-h photoperiod (60 μ$Es^{-1}m^{-2}$) for growing new plantlets for 5 yr. Leaves and stems aseptically excised from these plantlets of 7 clones were used as explants.

Infection, bacteria elimination, and induction

Agrobacterium tumefaciens strain A281 and *A. rhizogenes* strains AR1600 and ATCC15834 were used for infection. Prior to inoculation, a bacterial suspension was cultured on YEB nutrient medium (Van Larebeke et al. 1977) incubated on a shaker (200 rpm) at 28℃ in the dark for 18 h. Small segments of leaf (0.5×0.5 cm) and stem (0.5 cm) explants were immersed in fresh *Agrobacterium* strain culture suspensions (10^8 bacterial cells mL^{-1}) for 5 min, and redundant bacterial suspension was blotted off using sterile filter paper. Explants were then cultivated on 0.75% agar-solidified MS medium supplemented with 3% sugar (called M2 medium). After 48 h of incubation, explants were rinsed 4 times with sterile water for 2 min and immersed in 1 g.L^{-1} timetin for 1 h to eliminate bacteria. Infected tissues were subcultured on M2 medium containing 200 mg.L^{-1} timetin and were made bacteria-free by transferring to fresh medium weekly 4 times. Around 300 leaf and 300 stem explants for each *Agrobacterium* strain used were then transferred to antibiotic-free M2 medium. The nearly 300 explants were composed of 40~50 explants.clones^{-1} derived from *in vitro* cultures of the 7 clones.

Transgenic crown galls and hairy roots began to develop 40 d after infection. Transformation rates were calculated 60 d after infection. After 3 mo, crown galls were excised and then cultured on MS medium containing 30 g.L^{-1} sucrose, 100 ml.L^{-1} coconut milk, 100 m g.L^{-1} casein hydrolysate, and 7.5 g.L^{-1} agar (called M3 medium), while hairy roots were cultured on M1 medium. Both crown galls and hairy roots were subcultured monthly. All cultures were placed in continuous darkness at 25±2℃.

Genomic DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from *T. sumatrana* hairy roots and untransformed shoots according to the CTAB (hexadecy ltrimethylammonium bromide) method (Doyle and Doyle 1990). The presence of the *rolB* gene in putative ATCC15834/AR1600 transgenic crown gall or hairy root lines was confirmed by a PCR with the following primers, *rolB*1: 5'-ATG GATCCCAAATTGCTATTCCCCCACGA-3' and *rolB*2: 5'-TTAGGCTTCTTTCATTCG GTTTACTGCAGC-3' (Hamill et al. 1991). Specific primers for the *iaaM* (tryptophan monooxygenase) gene (M281-1: 5'-GGCTG GCCGATGGTCGCTTC-3' and M281-2: 5'-TCGCCCCCATTTCGGCCACG-3') were used to confirm the presence of foreign genes in putative A281 transgenic clones. Genomic 50~100 ng and 10 ng of plasmid DNA were used for the PCR amplification in a 20-uL reaction volume with a KAPA2G Fast Hotstart ReadyMix PCR kit (KAPA Biosystems, Wilmington, MA, USA). PCRs were performed using the following program of the PCR Thermal Cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA): initial denaturation at 95℃ for 3 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 65℃ (*rolB*) or 66℃ (*iaaM*) for 15 s, and extension at 72℃ for 20 s, with a final extension at 72℃ for 5 min prior to holding at 4℃. PCR-amplified products were subjected to electrophoresis in a 1% agarose gel followed by staining with ethidium bromide and destaining in double-distilled water. The

gel was then photographed using a gel documentation system (Vilber Lourmat, Marne La Vallée, France).

Culture of transformed crown galls and hairy roots

After 6 mon of cultivation of transgenic lines, 2 lines of crown galls (named G1 and G2), 2 lines of calli from hairy roots (named RC1 and RC2), and 4 lines of hairy roots (named HR1~4) from clone E7 (collected from Daisetsuzan), which produced a relatively high amount of biomass, were collected for a growth experiment. Transformed crown galls and calli derived from hairy roots of 0.8 g (fresh weight, FW) were cultured on M3 medium, while 0.5 g transformed hairy roots was cultured on M1 medium. To compare with the growth of transformed explants, 0.8 g (FW) untransformed stem calli were cultured on M3 medium with the addition of 2 $mg.L^{-1}$ α-naphathaleneacetic acid (NAA) (Chang et al. 1996), while 0.5 g of untransformed roots was cultured on M1 medium. Each treatment was replicated 3 times. All cultures were placed in continuous darkness at 25±2℃. After cultivating for 40 d, growth and taxane contents were recorded. The growth index was calculated as:

$$\frac{\text{Final FW - initial FW.}}{\text{Initial FW}}$$

To evaluate the potential of scaled-up culture, 1 g of crown gall 2 and 1.2 g of hairy root 4 were cultured in liquid M3 and M2 medium (50 ml per 250-ml flask), respectively, at 25℃ on a rotary shaker (75 rpm for crown galls and 60 rpm for hairy roots) in the dark with 3 replications. Cultures were harvested after 30 d, and the FW and taxane contents were measured.

Extraction and determination of taxanes

Each gall or hairy root sample (1~2 g FW) was freeze-dried and ground to a fine powder with a mortar and pestle. The powder was lyophilized and extracted with 10 ml methanol by sonication for 30 min. The methanolic extract was centrifuged for 5 min at 1000 xg. The residue was re-extracted with 10 ml methanol. Clear supernatants and the re-extract were combined and passed through a 0.2-μm filter (Acrodisc^{R} Lc 13 PVDF, Ann Arbor, MI, USA). The analysis using high-performance liquid chromatography (HPLC) was carried out on a Waters 600 system (Milford, MA, USA) equipped with a 717 plus 10-μl loop autosampler injector and a 2996 system photodiode-array detector using a Chromolith Performance RP-18e monolithic column (150×4.6 mm, 5-μm particle size) (Supelco Discovery, Bellefonte, PA, USA). The mobile phase for elution was 0.03 M ammonium acetate: acetonitrile (60: 40), at a flow rate of 1 $ml.min^{-1}$ and UV detection at 230 nm. Standard curves of 3 taxanes, i.e. 10-DB, BC, and paclitaxel, were obtained using authentic samples (Sigma-Aldrich, St. Louis, MO, USA) (Ho et al. 1997). Taxane concentrations in extracts were expressed as a percentage of dry weight (DW) of cultures. For DW measurements, residues were dried at 60℃ for 48 h after being extracted.

Statistical analysis

Means of the growth index of crown galls, roots, and calli of different lines were calculated and compared by Duncan's multiple-range test at the 5% level of significance using an analysis of variance (ANOVA) in SAS (vers, 8.2, SAS Institute, Cary, NC, USA).

RESULTS

Induction of crown galls and hairy roots of *T. sumatrana*

Explants of *T. sumatrana* leaves and

stems after coculturing with *Agrobacterium* and culturing on M2 medium with timetin for 1 mon were transferred into M2 medium without timetin and became swollen at week 2 on growth regulator-free media. However, some of them died, while some developed crown galls or hairy roots and continued growing, showing evidence of successful transformation. Timetin effectively eliminated *Agrobacterium* from stem and leaf explants by 97.1 and 98.6%, respectively. Explants inoculated with *A. tumefaciens* strain A281 formed crown galls, while those inoculated with *A. rhizogenes* strains AR1600 and ATCC15834 formed hairy roots or nodular calli. Results showed variations in transformation efficiencies among different *Agrobacterium* strains and plant parts (Table 1). Leaves were less susceptible to infection than stems, as only 30 of 887 non-contaminated leaves formed crown galls or nodular calli (1.70~5.76%), and no hairy roots formed. For transformation rates of hairy roots derived from stem explants among strains, ATCC15834 induced higher rooting rates than did AR1600 (15.09 vs 19.24%). Explants inoculated with strain A281 formed crown galls at the cut surfaces after 3~4 wk (Fig. 1A). Transformation rates of stem and leaf explants forming crown galls were 42.3 and 5.8%, respectively. At 6~8 wk after being inoculated with *A. rhizogenes*, hairy roots appeared either directly from stem explants (Fig. 1B) or from nodular calli which were initiated from stems or cut surfaces (Fig. 1C). Over 73% of hairy roots were induced from nodular calli. Strain ATCC15834 had a higher transformation rate than AR1600 (19.24 vs.15.09%). There were 4.2~7.6% of inoculated stem segments that formed only nodular calli without developing hairy roots.

Among all 7 clones, stem explants from clones E7 and NJR had the highest transformation efficiencies of both crown gall and hairy root formation (Table 2). A281 produced the highest percentages of crown galls (62.22 and 62.86%), whereas AR1600 and ATCC15834 produced the highest percentages of hairy roots 27.27~39.53%). The lowest transformation rates occurred in clones E2 and J85, the crown gall formation rates of which were 15~20%, and hairy root formation rates when inoculated with strain ATCC15834 were 6.7~7.5%. No hairy roots developed when inoculated with strain AR1600. Average transformation rates of hairy roots of AR1600 and ATCC15834 in Table 2 had the same integer values but a few differences in decimal places compared to total transformation rates in Table 1. These minor differences were caused by great variances in transformation

Table 1. Numbers and percentages of explants forming crown galls and hairy roots after being infected with *Agrobacterium tumefaciens* A281 and *A. rhizogenes* AR1600, and ATCC15834 of *Taxus sumatrana*

Explant	*Agrobacterium* strain	No. of explants	No. of explants formed			Percent of explants formed			
			Crown galls / nodular calli only	Nodular calli and hairy roots	Hairy roots only	Crown galls / nodular calli only	Nodular calli and hairy roots	Hairy roots only	Total hairy roots
Stems	A281	298	126	0	0	42.28	0.00	0.00	0.00
	AR1600	285	12	35	8	4.21	12.28	2.81	15.09
	ATCC15834	291	22	41	15	7.56	14.09	5.15	19.24
Leaves	A281	295	17	0	0	5.76	0.00	0.00	0.00
	AR1600	294	5	0	0	1.70	0.00	0.00	0.00
	ATCC15834	298	8	0	0	2.68	0.00	0.00	0.00

Table 2. Percentages of stem explants forming crown galls and hairy roots with *Agrobacterium tumefaciens* A281 and *A. rhizogenes* AR1600 and ATCC15834 from different clones of *Taxus sumatrana*

Cloness	Locatiions of Mother trees[1)]	Percent of explants formed				
		Crown galls	Nodular calli only		Hairy roots (from stem explant and nodular calli)	
		A281	AR1600	ATCC15834	AR1600	ATCC15834
E2	Daisetsuzan	20.00	2.38	0.00	0.00	7.50
E7	Daisetsuzan	62.22	6.67	11.63	35.56	39.53
R2	Dayuling	50.00	2.17	6.67	13.04	22.22
R5	Dayuling	47.62	2.50	7.50	17.50	20.00
NJR	Jalishan	62.86	3.33	15.15	33.33	27.27
J49	Piluchi	36.96	7.14	11.11	9.52	13.33
J85	Piluchi	15.00	5.00	2.22	0.00	6.67
Average transformation rates		42.09	4.17	7.75	15.56	19.50

[1)] Daisetsuzan is located at Taichung City, Dayuling in Haulien County, Jalishan in Miaoli County, and Piluchi in Nantou County.

rates among clones as shown in Table 2.

Characterization and growth of crown galls and hairy roots

Crown galls, looking more granular in texture than untransformed calli, grew slowly in the first 3 mon but subsequently grew rapidly (Fig. 1D). Compared to untransformed roots, hairy roots generally grew fast, showed plagiotropic root growth, and were greatly branched on plant growth regulator-free medium.

There were 4 kinds of morphologic types of *T. sumatrana* hairy roots: thin and dense (Fig. 1E), highly branched (Fig. 1F), with frequent calli (Fig. 1G), and with occasional calli (Fig. 1H). All types occurred in hairy roots induced from *A. rhizogenes* AR1600 and ATCC15834. However, the majority of hairy roots induced from strain ATCC15834 were of the highly branched type (55.4%), while those induced from strain AR1600 were of the thin and dense type (72.1%) (Table 3). Some hairy roots formed callus tissues, especially in the case of ATCC15834-induced hairy roots (21.4%). When the calli were cultivated on M3 medium favorable for crown gall culture, 90% grew, remainded as calli, and occasionally started rooting.

Crown galls and calli derived from hairy roots cultured on M3 medium grew faster than did untransformed stem calli cultured on M3 medium with growth regulators. Growth indexes among transgenic cell lines varied 4.9~5.5 after 40 d of culture, while that of untransformed calli, which did not grow without plant growth regulators, was only 2.5 on medium with growth regulators (Table 4). Moreover, calli derived from hairy roots grew faster than did crown galls. There were no differences in growth indexes between different lines of crown galls (G1 and G2) and calli (RC1 and RC2).

Growth rates of hairy roots were 12~16-times higher than those of untransformed roots (Table 4). After being cultured in M1 medium for 40 d, hairy roots increased 4.9~5.9-fold of the initial FW (growth index = 3.9~4.9). *Agrobacterium* strains did not affect the growth of hairy roots, but the form of the hairy root did, with highly

branched hairy roots growing faster than thin and dense ones.

For scaled-up culture potential evaluation, the FW of 1 g of crown galls (G2) after 30 d of culture increased to 9.13±0.32 g (growth index = 8.13), while that of 1.2 g of hairy root (HR4) increased to 8.43±0.21 g (growth index = 6.03) (Fig. 2). Both forms grew better in liquid medium than on solid medium (growth indexes = 5.1 and 4.9, respectively).

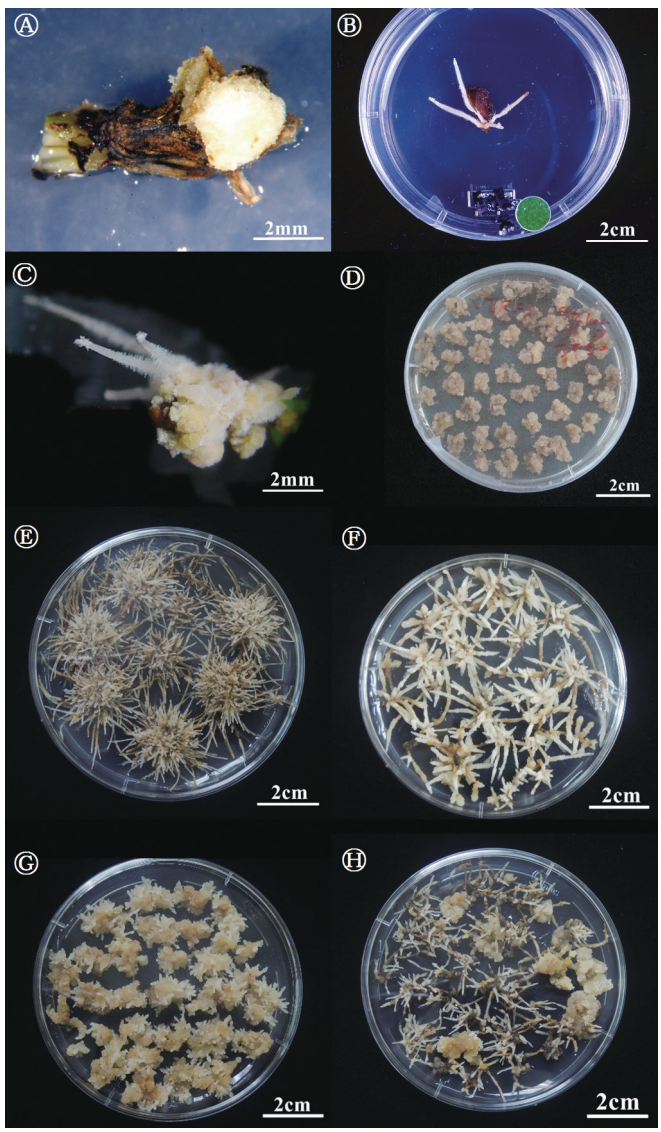

Fig. 1. Crown gall and hairy root formation (A-C) and morphologic types (D-H) of *Taxus sumatrana*. (A) Crown gall induced from a stem explant, (B) hairy roots induced from a stem explant, (C) hairy roots induced from nodular calli, (D) fast-growing crown galls, (E) thin and dense hairy roots, (F) highly branched hairy roots, (G) hairy roots with frequent calli, (H) hairy roots with occasional calli.

Verification of the transformed status of crown gall and hairy root culture

The PCR analysis revealed the existence of *Agrobacterium* genes in crown galls and hairy roots, including the presence of the *iaaM* gene (700 bp) in strain A281 transgenic clones (Fig. 3A), and the *rolB* gene (780 bp) in AR1600 (Fig. 3B) and ATCC15834 (Fig. 3C)

Table 3. Structure of hairy roots induced from *Agrobacterium rhizogenes* AR1600 and ATCC15834 of *Taxus sumatrana*

Agrobacterium rhizogenes	Hairy root lines	Structure of hairy roots (%)			
		Thin and dense	Highly branched	Formed lots of calli	Formed occasional calli
AR1600	43	72.09	11.63	9.30	6.98
ATCC15834	56	14.29	55.36	21.43	8.93

Table 4. Growth of *Taxus sumatrana* (E7) untransformed calli, roots, and transformed crown galls, hairy roots, and calli derived from hairy rootsafter 40 d in culture

Culture line	*Agrobacterium* strain	Final fresh weight (FW) (g)	Final dry weight (g)	Growth index[1)]
Untransformed calli	-	2.53±0.31	0.127±0.015	2.54[d]
G1 (crown galls from stems)	A281	4.43±0.12	0.288±0.008	4.92[ab]
G2 (crown galls from stems)	A281	4.57±0.40	0.274±0.024	5.08[ab]
RC1 (calli from hairy roots)	AR1600	4.83±0.25	0.242±0.013	5.42[a]
RC2 (calli from hairy roots)	ATCC15834	4.87±0.35	0.268±0.019	5.46[a]
Untransformed roots	-	0.65±0.04	0.062±0.004	0.31[e]
HR1 (thin and dense hairy roots)	AR1600	2.50±0.10	0.250±0.010	4.00[c]
HR2 (thin and dense hairy roots)	ATCC15834	2.45±0.21	0.196±0.016	3.91[c]
HR3 (highly branched hairy roots)	AR1600	2.73±0.21	0.205±0.016	4.47[b]
HR4 (highly branched hairy roots)	ATCC15834	2.97±0321	0.282±0.029	4.93[ab]

[1)] Values of the growth index defined as ((finial FW - initial FW)/ initial FW) followed by the same letter do not significantly differ at the 5% level by Duncan's test. Crown galls and calli initial FW = 0.8 g, roots initial FW = 0.5 g.

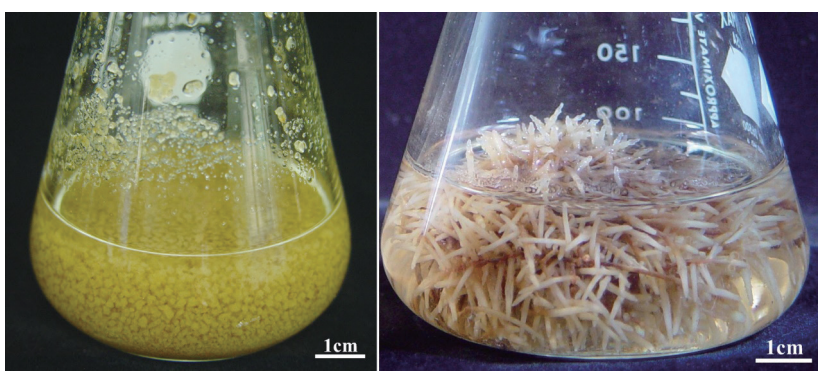

Fig. 2. Crown gall G2 (left) and hairy root HR4 (right) of *Taxus sumatrana* (E7) cultured in MS liquid medium without plant growth regulators for 30 d.

transgenic clones. No *iaaM* or *rolB* genes were detected in normal *T. sumatrana* parts.

Taxane contents in crown galls and hairy roots

Transformed crown galls, calli, and hairy roots had higher contents of taxanes (i.e., 10-DB, BC, and paclitaxel) than did untransformed ones (Fig. 4). Transformed crown galls and calli produced more taxanes than did hairy roots. Taxane contents varied in different culture lines, but not with *Agrobacterium* strains. Production levels of BC and paclitaxel were similar, and higher than those of 10-DB. For crown galls, calli, and hairy roots, 10-DB contents were 1.3~5.2, 1.2~3.6, and 0.4~1.8 ppm; BC contents were 252~350, 250~385, and 28~128 ppm; and paclitaxel contents were 286~298, 36~320, and 25~165 ppm, respectively.

DISCUSSION

In plant biotechnology, the genetic transformation of *Taxus* plants requires much

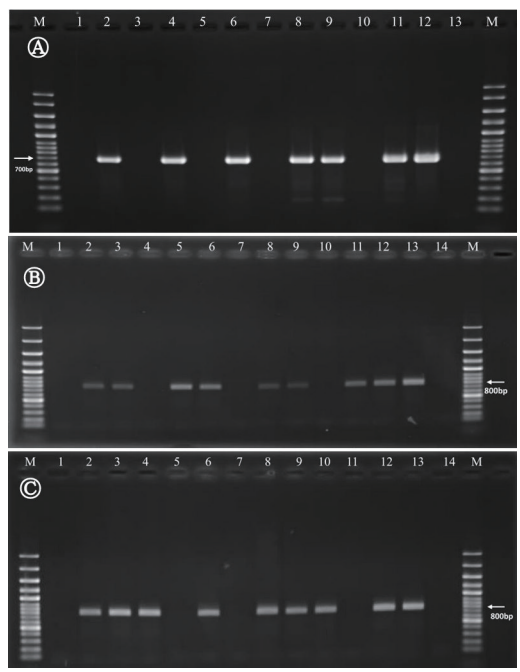

Fig. 3. Molecular verification of transgenic lines of T*axus sumatrana* by a PCR. (A) A281 (*iaaM* gene): lanes 1, 3, 5, 7, 10 from clones E7, R2, R5, NJR, and J49, respectively, were untransformed shoots; lane 2 from clone E7A1, lane 4 from R2A3, lane 6 from R5A1, lanes 8 and 9 from NJRA3 and NJRA4, lane 11 from J49A1, lane 12 from A281 (positive control), and lane 13 was the NTC (no-template control). (B) AR1600 (*rol B* gene): lanes 1, 4, 7, and 10 from E7, R2, R5, and NJR (untransformed shoots), lanes 2 and 3 from E7B1 and E7B8, lanes 5 and 6 from R2B11 and R2B16, lanes 8 and 9 from R5B3 and R5B15, lanes 11 and 12 from NJRB16 and NJRB19, lane 13 from R1600 (positive control), and lane 14 was the NTC. (C) ATCC15834 (*rol B* gene): lanes 1, 5, 7, and 11 from E7, R2, R5, NJR (untransformed shoots), lanes 2, 3, and 4 from E7C1, E7C3, and E7C7, lane 6 from R2C3, lanes 8, 9, and 10 from R5C1, R5C3, and R5C5, lane 12 from NJRC2, lane 13 was ATCC15834 (positive control), and lane 14 was the NTC.

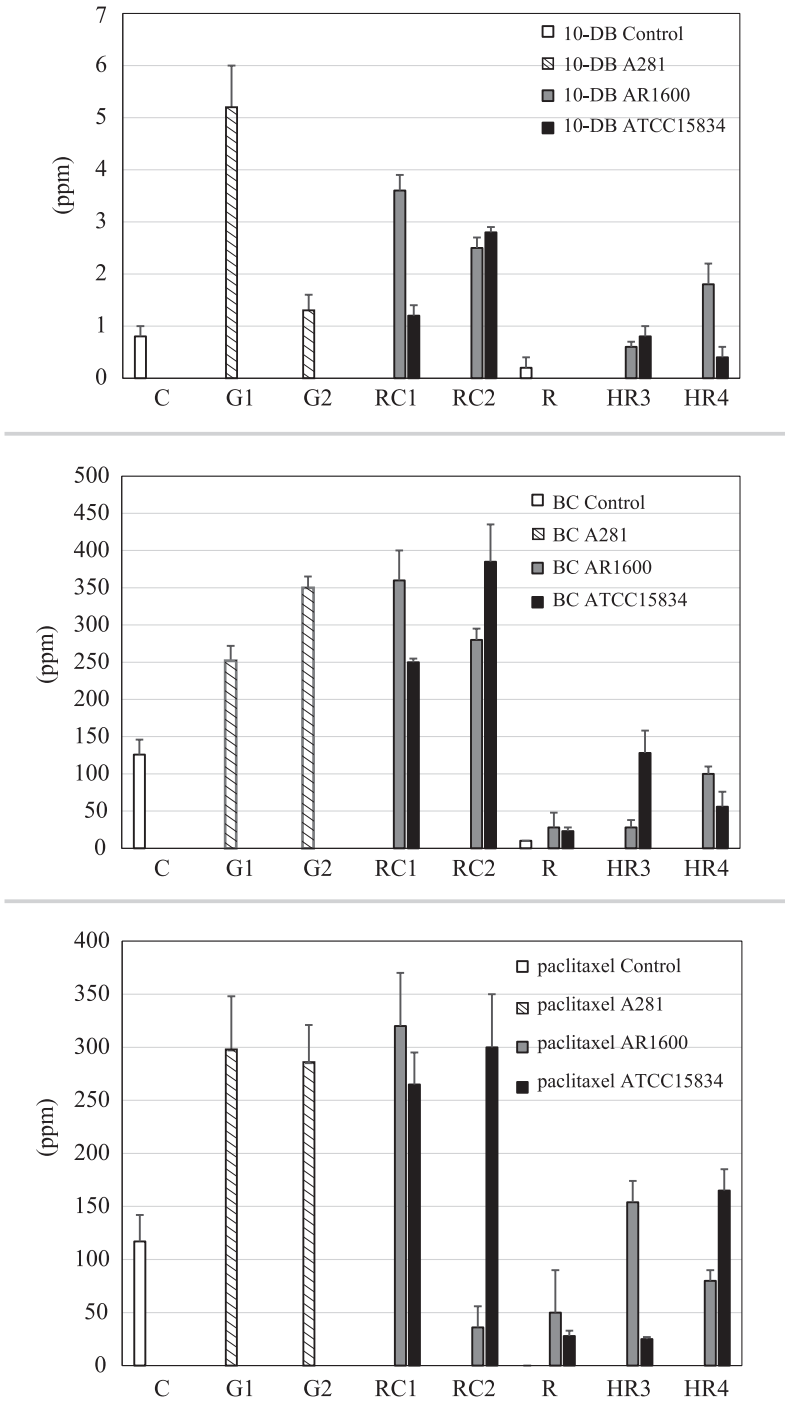

Fig. 4. Taxane contents (ppm = mg.kg^{-1} dry weight) in different culture lines of *Taxus sumatrana* (E7) induced by A281, AR1600, and ATCC15834. C, untransformed stem calli; G1 and G2, crown galls; RC1 and RC2, calli formed from hairy roots; R, untransformed roots; HR3 and HR4, hairy roots; 10-DB, 10-deacetylbaccatin III; BC, baccatin III.

effort. Only a few successful cases using an *Agrobacterium* system or biolistic techniques obtained transformed cell cultures of *Taxus* (Exposito et al. 2010). The *Agrobacterium* strain, plant species, and explant sources influence the transformation rate of *Taxus* plants. In a study by Han et al. (1994), *A. tumefaciens* strains Bo542 and C58 were used to inoculate stem explants of mature *T. baccata* and *T. brevifolia*. Strain Bo542 induced more galls than did strain C58 (24 vs. 4%). *Taxus brevifolia* was less susceptible to *Agrobacterium* than *T. baccata* (7 vs. 14%). Among the 3 *A. rhizogenes* strains of ATCC15834, TR105, and LBA9402 that Furmanowa and Syklowska-Baranek (2000) used to examine the induction of hairy roots from stem explants of *T x media*, strain LBA9402 was the only active one (transformation rate = 3%). Kim et al. (2009) reported that *A. rhizogenes* strains R1000 and ATCC15834 successfully induced hairy roots in *T. cuspidata* (with transformation rates of 26.4 and 25.5%, respectively), whereas the A4 strain failed to generate any hairy roots. In our study, both *Agrobacterium* strains and *Taxus* clones affected the transformation efficiency and transgenic strain. Strain A281 had the highest transformation ability, followed by AR1600 and ATCC15834. Hairy root formation rates of infection with strains ATCC15834 and AR1600 were 19.2 and 15.1%, respectively.

Challenges of establishing *Agrobacterium*-mediated transformation systems for *Taxus* species include low crown gall and hairy root induction rates, slow growth at the beginning, and successive culture difficulties. Han et al. (1994) reported that tumors of *T. baccata* began to grow rapidly in month 4. Hairy roots of *T. x media* barely grew in the first year, but stably grew after culturing for 1.5 yr (Furmanowa and Syklowska-Baranek 2000). In a study by Kim et al. (2009), although 107 hairy root lines were induced from *T. cuspidata* seedlings, including 72 lines infected with AR1000 and 35 lines infected with ATCC15834, only 3 lines infected with R1000 were able to steadily grow on plant growth regulator-free medium. Exposio et al. (2010) established cell cultures from calli, induced from hairy roots of *T. x media* on medium with growth regulators. In this study, crown galls and hairy roots of *T. sumatrana* induced by infection with strains A281, AR1600, and ATCC15834 could grow on plant growth regulator-free medium. Crown galls derived from the A281 strain grew slowly initially, but formed a few more-rapidly growing cells after being cultured for 3 mon. A similar result of crown galls in *T. baccata* was reported by Han et al. (1994). However, hairy roots generated from the AR1600 and ATCC15834 strains grew well. We found that some hairy roots produced calli, which was also observed in hairy root culture of *T. x media* (Furmanowa and Syklowska-Baranek 2000). The simultaneous production of both hairy roots and calli might result from the active expression of *rol* genes due to the presence *vir* genes on the *A. rhizogenes Ri*-plasmid which causes extreme synthesis of endogenous auxin and cytokinin in host cells. (Setamam et al. 2014).

Owing to its better genetic stability than callus and cell culture, hairy root culture is a novel source for valuable secondary metabolite production (Hu and Du 2006, Sharma et al. 2013). Our results demonstrated a great variation among transgenic strains in taxane contents of induced transgenic cells and hairy roots of *T. sumatrana*. This observation is also in agreement with results of callus cultures derived from *T. sumatrana* explants (Chang et al. 1996). Elicitors, such as methyl jasmonate, coronatine, and cyclodextrins, can

effectively increase the taxane production of nontransgenic cells (Sabater-Jara et al. 2010, Cusido et al. 2014), as well as that of hairy root cultures (Furmanowa and Syklowska-Baranek 2000, Kim et al. 2009, Syklowska-Baranek et al. 2009, Exposito et al. 2010) in *Taxus* species. Developments in the ongoing search for effective elicitation treatments will increase the paclitaxel production of transgenic cell crown galls, hairy roots and calli from hairy roots.

CONCLUSIONS

This is the first report on establishing crown gall and hairy root cultures of *T. sumatrana*. We successfully transformed *A. tumefaciens* strain A281, and *A. rhizogenes* strain AR1600 and ATCC15834 genes into *T. sumatrana*, which was verified by a PCR. The induced crown galls and hairy roots, and calli derived from hairy roots were able to grow on plant growth regulator-free medium. According to the HPLC analyses, taxane (10-DB, BC, and paclitaxel) contents in transgenic cells were higher than those of untransformed cells and grew rapidly in liquid medium. This system is able to provide a stable supply for clinical taxane applications. Due to taxane contents variingwith culture lines and not being related to *Agrobacterium* strains, we suggest a two-step protocol to select suitable transgenic lines for scale-up cultures: the first step is to obtain more transgenic cultures by infecting potential *Taxus* clones with *A. rhizogenes* strain ATCC15834, and the next step is to select superior lines with better growth indexes and taxae contents. For scaled-up culture, we suggest that more research on high-taxane hairy root line selection, suitable elicitors, culture methods and conditions, and taxane-producing related gene studies in the future are necessary.

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